The importance of timing differentiation during limb muscle development

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Background: Skeletal muscle of trunk, limbs and tongue develops from a small population of cells that originates from somites. Although promoters and inhibitors of muscle differentiation have been isolated, nothing is known about how the amplification of the muscle precursor pool is regulated; this amplification provides muscle mass during development. Furthermore, little is known about how cells accumulate in the pre-muscle masses in the limbs. We investigated the role of bone morphogenetic proteins (BMPs) and Sonic hedgehog (Shh) during proliferation, differentiation and positioning of muscle.

Results: The proliferation of muscle precursors in limbs was linked to *Pax-3* expression. Ectoderm removal downregulated *Pax-3* expression, arrested proliferation and prematurely initiated muscle differentiation which exhausted the muscle precursor pool and prevented further muscle growth. BMP-2, BMP-4 and BMP-7 had a dose-dependent effect on pre-myogenic cells: low concentrations maintained a *Pax-3*-expressing proliferative population, substituting for ectoderm-derived proliferative signals and delaying differentiation, whereas high concentrations prevented muscle development, probably by inducing apoptosis. In the limb, Shh upregulated *Bmp-2* and *Bmp-7* expression which delayed muscle differentiation, upregulated *Pax-3*, amplified the muscle precursor population and stimulated excessive muscle growth.

Conclusions: These data indicate that embryonic muscle growth requires muscle differentiation to be delayed. Muscle differentiation may occur through a default pathway after cells escape proliferative signals. Positioning of muscle is regulated by high concentrations of BMPs, thus a single type of signalling molecule can determine crucial steps in muscle development: when and where to proliferate, and when and where to differentiate.

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Background

An increase in skeletal muscle mass and many different muscles were required to adapt paired appendages to terrestrial life during the evolution of tetrapods. Whereas fish move their paired fins with muscle derived from extended myotomes, a different mechanism is used by tetrapods [1–3]. To generate the necessary amount and diversity of limb muscle, tetrapods have recruited a few committed but dividing myogenic precursor cells from the dermomyotome which migrate into the limb [4]. During limb development, these muscle progenitors proliferate, differentiate and are patterned according to cues from their new environment [5,6]. These observations have raised two fundamental questions: how is muscle growth generally controlled and how is muscle positioned correctly?

Myogenesis consists of three overlapping phases: determination, proliferation and differentiation [7]. Before any myogenic determination has occurred, most cells of a pre-streak epiblast-stage embryo possess myogenic competence, and when these cells are dispersed and grown in culture they express muscle-specific proteins [8]. However, the muscle phenotype is only expressed in a subset of cells which originate from somites, paraxial head mesoderm and prechordal plate mesoderm relatively late during development [9,10]. Grafting experiments have established that cells of the dorsal half of the somite become determined to a muscle fate approximately 6 hours after somite formation [9]. Further development of somite-derived muscle is dependent on the position of the precursors within the dorsal somite half. Cells from the dorsomedial somite give rise to the epaxial muscle (intrinsic back muscle), whereas the hypaxial muscle (ventral body, limb and tongue muscle) originates dorsolaterally [11]. Additionally, epaxial and hypaxial muscle differ in the time period between their determination and the onset of differentiation; epaxial muscle cells start to differentiate a few hours after determination, whereas hypaxial muscle cells remain undifferentiated for over 2 days [12,13].

The onset of differentiation is dependent on extrinsic signalling from adjacent tissues [9]. Signals from the neural

tube and notochord can induce muscle differentiation in cells of the dorsomedial somite quarter, whereas signals from the lateral plate prevent differentiation in the dorsolateral somite quarter [14–18]. Little is known about how and why limb-muscle precursors are maintained for such a long period in an undifferentiated state. A clue has emerged recently from work by Pourquié *et al.* [19] who found that bone morphogenetic protein 4 (BMP-4) is responsible for maintaining the undifferentiated state of prospective hypaxial muscle and hence counteracts the differentiation-inducing activity of the neural tube. When limb mesenchyme is exposed to high levels of BMPs, however, muscle cells are eliminated, probably by induction of apoptosis [20]. Limb muscle originates from cells that have migrated as undifferentiated precursors from the somites. These muscle precursor cells accumulate in dorsal and ventral mesenchyme by stage 20 and form two blocks that extend distally during limb outgrowth [21]. The mechanism by which myogenic cells are localised into the dorsal and ventral limb mesenchyme has yet to be determined, however.

We have investigated how a relatively small number of determined myogenic cells are amplified to become a large muscle mass and how muscle is positioned during development. We show that surface ectoderm maintained a population of proliferating undifferentiated muscle precursor cells (characterised by the expression of the transcription factor Pax-3) in the underlying mesenchyme. When ectoderm was removed, myogenic cells downregulated *Pax-3* expression and stopped proliferating. Ectoderm removal resulted in an upregulation of the gene encoding the transcription factor MyoD (the first genetic evidence of cells entering the myogenic programme) and muscle began to differentiate. This prematurely depleted the proliferative precursor reservoir and resulted in muscle loss. BMPs 'at low concentration' could mimic ectoderm activity, resulting in amplification of myogenic precursors and in increased muscle mass. Our data suggest that the muscle-promoting activity of the signaling molecule Sonic hedgehog (Shh) is probably indirect and is mediated by BMPs. Exposure to high concentrations of BMP may lead to muscle loss. A dose-dependent response of myogenic cells to BMP may spatially co-ordinate growth (through proliferation) and positioning (through apoptosis). Differentiation of muscle eventually occurs after cells escape the influence of BMP signalling, through a default pathway.

Results

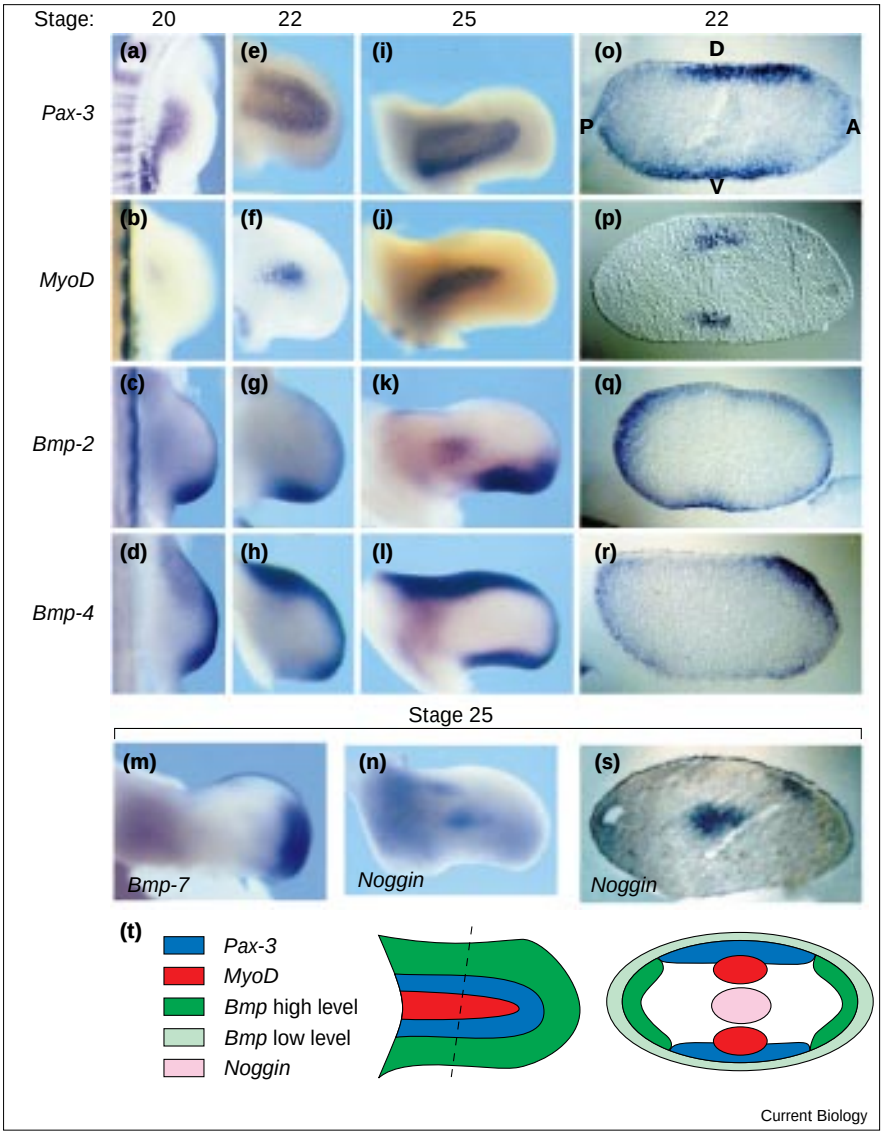
Spatial relationship of the expression domains of muscle markers during limb development

Pax-3 expression has been proposed to be a marker for undifferentiated myogenic cells and *MyoD* expression a marker for differentiating muscle cells [7,22]. We examined the spatial distribution of transcripts of these genes during wing development of chick embryos by *in situ* hybridisation. Myogenic precursors that express *Pax-3* accumulated as pre-muscle masses in the ventral and dorsal limb mesenchyme by stage 20 (Figure 1a). The first cells expressing *MyoD* were just detectable in the proximal centre of the pre-muscle masses at this stage (Figure 1b). By stage 22, the *Pax-3* expression domain extended distally (Figure 1e), but did not extend in a central region that correlated to the position of *MyoD* expression (Figure 1f). Thus, myogenic cells are organised in a sequence of expression domains during limb development, with undifferentiated cells expressing *Pax-3* situated anteriorly, posteriorly and distally to the differentiating cells expressing *MyoD* in the proximal centre of the pre-muscle masses (Figure 1t).

We examined transverse sections of the wing buds and found that the pre-muscle masses were organised into a subectodermal layer that expressed *Pax-3* (Figure 1o) and a layer of *MyoD*-expressing cells situated towards the mesenchymal core of the limb (Figure 1p). Therefore, myogenic cells are organised in a sequence of *Pax-3* and *MyoD* expression domains in all three limb axes. This spatial relationship between *Pax-3* and *MyoD* expression was maintained during further limb outgrowth (Figure 1i,j) up to stage 26–27, after which muscle cells were reorganised during the process of muscle individualisation.

We examined the distribution of signalling molecules that could be responsible for the spatial arrangement of *Pax-3* and *MyoD*. *Pax-3*-expressing cells were never detected in the margins of the early limb (Figure 1a,e,i). At stage 20, *Bmp-2* was expressed in the posterior limb margin and in the apical ectodermal ridge (AER; Figure 1c). *Bmp-4* was expressed in anterior, posterior and distal limb margins and in the AER (Figure 1d). This expression pattern was maintained during further limb outgrowth (Figure 1g,h,k,l), except that by stage 25 *Bmp-7* was expressed in distal wing tissue (Figure 1m). Thus, cells expressing *Bmp-2*, *Bmp-4* and *Bmp-7* surround the *Pax-3* expression domain of the dorsal and ventral pre-muscle masses (Figure 1t). Transverse sections of stage 22 limbs revealed strong expression of *Bmp-2* in posterior mesenchyme and strong expression of *Bmp-4* in anterior mesenchyme; *Bmp-4* was expressed less in posterior mesenchyme (Figure 1q,r). *Bmp-2* was expressed at low levels in dorsal ectoderm and more prominently in ventral ectoderm, as well as at low levels in dorsal and ventral sub-ectodermal mesenchyme. *Noggin*, a gene which encodes an antagonist of BMP [23], was expressed in the mesenchymal core of the developing wing bud and so was in close proximity to the *MyoD*-expressing cells (Figure 1n,s). There was therefore an ordered array of gene expression in the limb: *Bmp-2* expression localised to the ectoderm, *Pax-3* expression in the sub-ectodermal layer, *MyoD* in a deeper mesenchymal layer and *Noggin* in a central core region (Figure 1t).

Figure 1



Expression of *Pax-3*, *MyoD*, *Bmp-2*, *Bmp-4*, *Bmp-7* and *Noggin* in wing buds: dorsal view of chick embryo whole-mounts. **(a–d)** Stage 20; **(e–h)** stage 22; **(i–n)** stage 25. (a,e,i) Expression of *Pax-3* transcripts revealed by *in situ* hybridisation; (b,f,j) expression of *MyoD*; (c,g,k) expression of *Bmp-2*; (d,h,l) expression of *Bmp-4*; (m) expression of *Bmp-7;* (n) expression of *Noggin.* **(o–r)** Stage 22 transverse sections from medial wing; posterior (P) on left, anterior (A) on right, dorsal (D) on top and ventral (V) on bottom. (o) *Pax-3* expression; (p) *MyoD* expression; (q) *Bmp-2* expression; (r) *Bmp-4* expression. **(s)** Stage 25 transverse section from medial wing showing *Noggin* expression. **(t)** Diagram summarising gene expression in a whole-mount of an embryo at stage 25. The right-hand panel shows a section of the whole-mount taken at the position indicated by the dotted line.

These observations led us to ask the following questions. Firstly, do tissues adjacent to *Pax-3*-expressing cells maintain *Pax-3* expression and do BMPs maintain this *Pax-3* expression? Secondly, do myogenic cells express *MyoD* only after escaping the influence of signals that maintain *Pax-3* expression? And thirdly, what are the consequences for muscle growth of prolonging the duration of *Pax-3* expression by myogenic cells or of inducing differentiation prematurely?

Limb-ectoderm-derived signals inhibit myogenesis by maintaining cells in a proliferative state

To determine whether signals from the ectoderm are important for the spatial organisation of cells expressing *Pax-3* and *MyoD* in the pre-muscle masses of the limb, we removed dorsal wing ectoderm from embryos at stages 21–23 and monitored the effect on the expression of *Pax-3* ($n = 10$) and *MyoD* ($n = 8$). A complete loss of *Pax-3* expression in the dorsal pre-muscle mass was detected 20 hours after ectoderm removal (Figure 2a,c,d). This shows that *Pax-3* expression may depend on ectodermal signals. We examined the fate of the myogenic cells after ectoderm removal and, surprisingly, detected not only an upregulation of *MyoD* but also an enlargement of the *MyoD* expression domain after only 10 hours (Figure 2b,e,f). Expression of *Pax-3* has been suggested to indicate that cells are in a proliferative state; we examined this by labelling limb cells with bromodeoxyuridine (BrdU) 17 hours after removing the ectoderm from stage 22–23 embryos ($n = 2$). Dorsal wing mesenchyme from which the overlying ectoderm had been removed showed almost no BrdU incorporation (Figure 2h) compared with

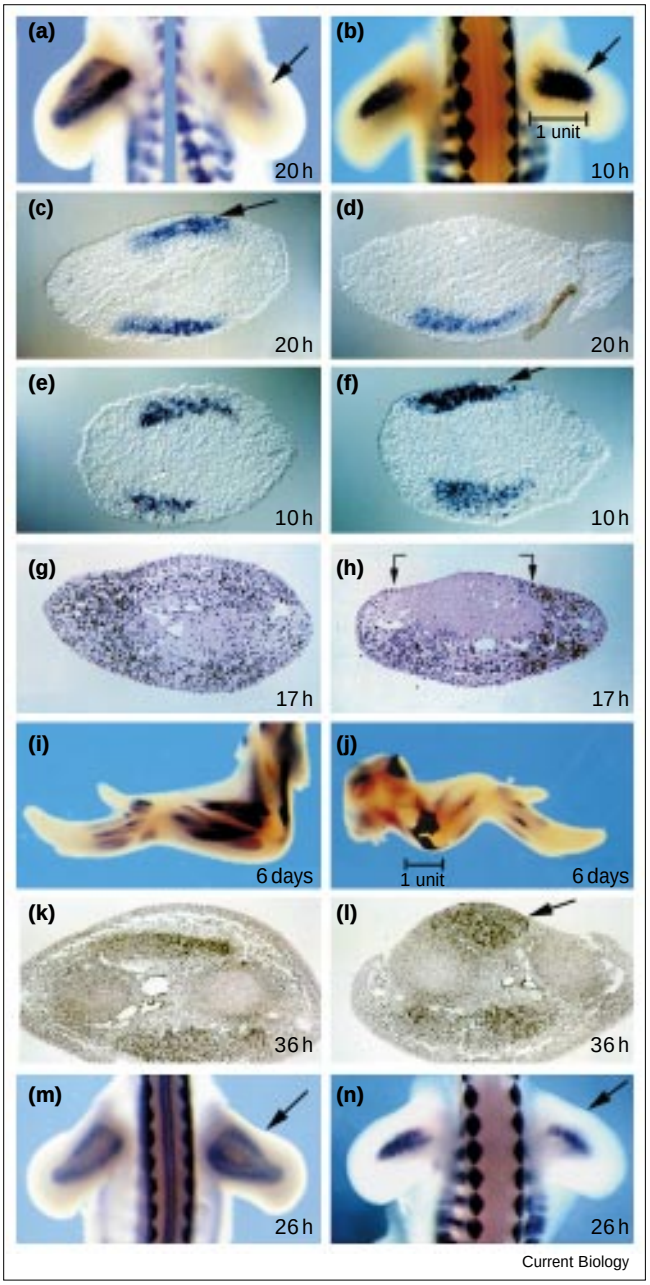


Figure 2

The influence of ectoderm on gene expression and cell proliferation in the limb. The time after manipulation is indicated; the operated side is on the right and the view is from dorsal side unless otherwise stated. (a) *In situ* hybridisation with a *Pax-3* probe after removal of the dorsal wing ectoderm at stage 22. The arrow indicates the dorsal pre-muscle mass. The shadow marks the expression in the ventral mass. (b) *MyoD* expression after ectoderm removal at stage 23. The arrow indicates upregulated expression and the size of the expression domain is marked as 1 unit. (c) *Pax-3* expression (arrow) in a transverse section of the control limb from (a). (d) *Pax-3* expression in transverse section of limb from (a) after ectoderm removal. (e) Transverse section of the embryo in (b) without ectodermal removal showing expression of *MyoD*. (f) Transverse section of the embryo in (b) after ectoderm removal showing *MyoD* expression (arrow). (g) BrdU incorporation in a transverse section of a normal stage 25 wing bud at medial level. (h) BrdU incorporation at stage 25 after dorsal ectoderm removal at stage 23 (extent of ectodermal removal indicated by arrows). (i) Expression of *MyoD* in 6 day wing. (j) Expression of *MyoD* in a 6 day wing after ectoderm removal at stage 21; 1 unit corresponds to the unit in (b). (k) Normal expression of muscle actin at a proximal region at stage 28. (l) Muscle actin expression (domain indicated by the arrow) 36 h after dorsal ectoderm removal at stage 24. (m) *Pax-3* expression (arrow) after grafting of dorsal zeugopod ectoderm of a stage 28 donor into the wing mesenchyme of a stage 20 host. (n) *MyoD* expression (arrow) after grafting of dorsal zeugopod ectoderm of a stage 28 donor into the wing mesenchyme of a stage 20 host.

the intact ventral side or unoperated contralateral wing (Figure 2g). Thus, there is a strong correlation between the expression of *Pax-3* and the proliferation of muscle precursors: the limb ectoderm maintains the pre-muscle mass in a proliferative state and following its removal, myogenic cells differentiate.

We followed the late muscular phenotype 6 days after ectoderm removal ($n = 9$) and found that the *MyoD* expression domain had not extended distally and the zeugopod and autopod were free of *MyoD* expression (Figure 2i,j), but the size of the residual proximal *MyoD* expression domain was comparable to the size of *MyoD* expression domain seen 10 hours after ectoderm removal (Figure 2b,j). Examination of the limb transverse sections (Figure 2j) after 6 days revealed that dorsal tissue in both the autopod and zeugopod were devoid of *MyoD* expression (data not shown). Most *MyoD* expression was detected at the 'elbow' region. In all limb ectoderm ablation experiments we left intact the ectoderm overlying limb margins and the zone of polarising activity (ZPA), which ensured further limb outgrowth and normal proximo-distal and antero-posterior patterning of the skeletal elements. The stylopod and zeugopod were shortened in length in all cases examined, however (Figure 2j). Serial sections revealed that the limb ectoderm had not regenerated over the ablated regions (the prospective stylopod and zeugopod) even after 5 or 6 days re-incubation, whereas the autopod was covered by ectoderm.

Expression of muscle actin showed that removal of ectoderm resulted not only in premature upregulation of *MyoD* expression but also in the myogenic programme being carried through to terminal differentiation. Expression of muscle actin on the non-operated side was confined to a thin deep layer (Figure 2k). On the operated side, however, there was greater expression of muscle actin and the expression domain extended more dorsally than in non-operated limbs (Figure 2l). Desmin expression was determined in limbs that were allowed to develop for 5 days after ectoderm removal at stage 22–23. Transverse sections revealed a similar expression pattern to that of *MyoD*; the dorsal tissues in both the autopod and zeugopod were devoid of desmin expression and desmin was only detected in the dorsal region of the elbow (data not

shown). We next performed reciprocal experiments whereby ectoderm from the dorsal wing zeugopod of a stage 28 donor was grafted into the middle of the dorsal wing mesenchyme of a stage 20 host and re-incubated for 26 hours ($n = 4$). In the presence of additional ectoderm, the *Pax-3* expression domain was expanded and wing buds appeared larger (Figure 2m) whereas identical grafting experiments showed reduced *MyoD* expression ($n = 6$) compared with contralateral limb (Figure 2n).

These results suggest that the ectoderm maintains a pool of *Pax-3*-expressing proliferating muscle precursors from which cells continually leave to form musculature of the limb. *Pax-3*-expressing cells that do not receive the proliferative signal spontaneously upregulate *MyoD* and enter the differentiation programme. Ectoderm removal exhausts the proliferative cell pool, and even though there is an initial burst of muscle differentiation, subsequent muscle growth is arrested due to a lack of precursors. Furthermore, the proliferative state of myogenic precursors seems to be linked to a migratory potential of these cells, because loss of proliferative activity after ectoderm removal prevented formation of distal muscle during further limb outgrowth.

BMP-2, BMP-4 and BMP-7 rescue *Pax-3* expression after ectoderm removal in the limb

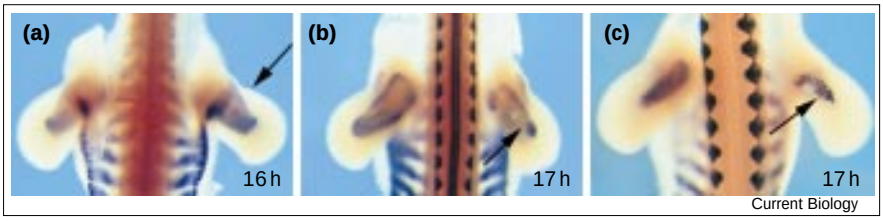
In early limb buds, the close spatial relationship between *Bmp* and *Pax-3* expression suggested that BMPs could maintain *Pax-3* expression in the same way that BMP-4 regulates *Pax-3* during early somite development [19]. To determine whether BMPs affect *Pax-3* expression in limbs, we implanted beads soaked in low concentrations of BMP-2 (1 μg/ml) into the wing mesenchyme of embryos at stage 22 ($n = 3$). *Pax-3* expression was upregulated in both dorsal and ventral pre-muscle masses 16 hours after this procedure (Figure 3a). Transverse sections of such limbs showed high *Pax-3* expression within the region of pre-muscle mass but no ectopic expression in other regions (data not shown). To determine how BMP-2 signalling is related to ectodermal signalling, we removed dorsal wing ectoderm of stage 22–23 embryos and applied beads soaked in BMP-2 into the dorsal wing mesenchyme and re-incubated the operated embryos for 17 hours ($n = 7$). BMP-2 was able to maintain *Pax-3* expression in the absence of ectoderm (Figure 3b, compare with Figure 2a). Furthermore, the premature upregulation of *MyoD* that normally follows ectoderm removal was not seen after BMP-2 bead implant (Figure 3c, compare with Figure 2b). Beads soaked in both BMP-4 (1 μg/ml; $n = 8$) and BMP-7 (1–100 μg/ml; $n = 23$) could also maintain *Pax-3* expression and prevent the premature upregulation of *MyoD* after ectoderm removal (data not shown). Another member of the family to which BMPs belong, TGF-β1 (1 μg/ml–1 mg/ml), could not maintain *Pax-3* expression after ectoderm removal, however (data not shown). Therefore, BMP-2, BMP-4 and BMP-7 induce and maintain *Pax-3* expression in the absence of ectoderm. Application of beads soaked in BMP-2 at 10 μg/ml and higher concentrations inhibited both *Pax-3* and *MyoD* expression, whereas BMP-2 at 100 ng/ml or less gave an equivalent effect to ectoderm removal alone (data not shown).

We tested whether ectoderm removal interferes with *Bmp-2* and *Bmp-4* expression in the mesenchyme of limb margins and in subectodermal mesenchyme ($n = 5$), but we found no alteration in the expression of either gene at these sites. Therefore BMP-2, BMP-4 and BMP-7 can upregulate *Pax-3* after being applied to the limb or can maintain *Pax-3* expression in the absence of ectoderm. BMPs expressed in the mesenchyme, however, are not able to substitute for the ectoderm but may enhance ectodermal proliferative action on myogenic cells. We found transcripts of *Noggin* in the limb core, suggesting that Noggin protein could antagonise the action of BMPs locally and induce muscle differentiation. This would result in the sequential distribution of *Pax-3*- and *MyoD*-expressing cells in all three limb axes described in Figure 1.

BMPs determine positioning of pre-muscle masses in the limb

High levels of BMPs have been implicated previously in preventing muscle development, possibly through the induction of apoptosis [20]. This is partly substantiated by expression data presented in this study showing that pre-muscle masses are positioned between the high levels of BMP-2, BMP-4 and BMP-7 at the anterior, posterior and distal limb margins. We implanted beads soaked in BMPs at high concentrations into the wing-bud mesenchyme of embryos at stages 21–22 and determined the effect on

Figure 3

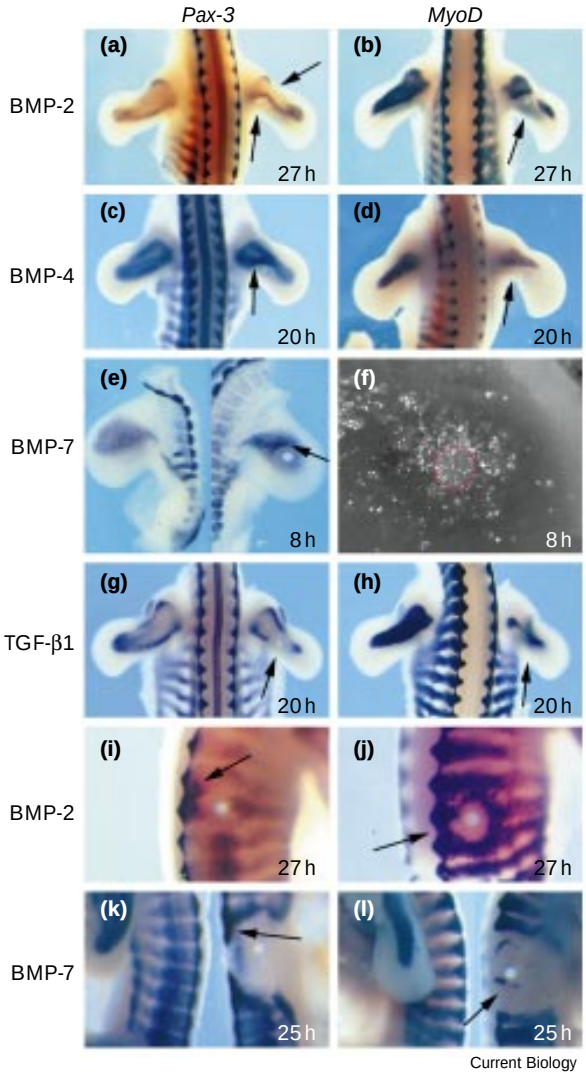


Effect of BMP-2 on limb-muscle development. (a) *Pax-3* expression (arrow) 16 h after implantation of a bead soaked in BMP-2 (1 μg/ml) into the wing mesenchyme of an embryo at stage 22. (b) *Pax-3* expression (arrow) and (c) *MyoD* expression (arrow) 17 h after the BMP-2 bead was implanted following dorsal ectoderm removal at stage 23.

expression of *Pax-3* and *MyoD* (BMP-2, $n = 12$; BMP-4, $n = 21$; BMP-7, $n = 14$). Whereas low concentrations of BMP-2 (1 μg/ml) enhanced *Pax-3* expression, BMP-2 at higher levels (100 μg/ml) prevented expression of *Pax-3* (Figure 4a) and *MyoD* (Figure 4b) close to the bead after 27 hours of re-incubation. Interestingly, we detected a contour of high *Pax-3* expression at some distance from the bead (Figure 4a). Similarly, implantation of BMP-4 beads (10 μg/ml) prevented expression of *Pax-3* (Figure 4c) and *MyoD* (Figure 4d) close to the bead, and again high *Pax-3* expression was detected at some distance from the bead (after 20 hours re-incubation). Finally, BMP-7 beads (1 mg/ml) induced apoptosis after only 8 hours (Figure 4f), and when the same wings were processed for *in situ* hybridisation against *Pax-3* transcripts we found a simultaneous downregulation of *Pax-3* expression around the bead (Figure 4e). The area of apoptosis appeared to be the same as the area of *Pax-3* loss. These effects were similar after 24 hours and the zone of high *Pax-3* expression bent around the bead (data not shown). We were unable to detect apoptosis at any time after implantation of beads soaked in lower concentrations of BMP-7 (100 μg/ml; data not shown). Beads soaked in either BMP-2 (100 μg/ml) or BMP-4 (10 μg/ml) were able to induce apoptosis 24 hours after being inserted into limbs from stage 21–22 embryos. Histological examination (using Feulgen-stained wax sections and toluidine-blue-stained semi-thin sections) of tissue architecture following implantation of beads soaked in high concentrations of BMPs showed little signs of necrosis (for example, cell debris or cellular aggregation). There were normal spindle-shaped cells next to and on the surface of the BMP-soaked beads, however. Histological examination after implantation of beads soaked in buffer (PBS) revealed no changes in tissue architecture (data not shown).

To determine whether the effect induced by the BMPs was specific to these proteins, we implanted beads soaked in TGF-β1 (10 μg/ml), and found that although expression of *Pax-3* (Figure 4g) and *MyoD* (Figure 4h) was prevented near the bead, there was no upregulation of *Pax-3* further away. In the limb, however, inserting beads with high concentrations of BMP might alter the migration of muscle precursors. We therefore introduced BMP-2 beads (100 μg/ml) into somites at thoracic level at stages 21–22, at which stage *Pax-3* and *MyoD* were expressed already, and re-incubated them for 27 hours ($n = 10$). We found that *Pax-3*-positive (Figure 4i) and *MyoD*-positive (Figure 4j) cells were lost near the bead but, remarkably, *Pax-3* was upregulated some distance away as in the limb (Figure 4i). Similarly, following BMP-7 bead insertion (1 mg/ml) into thoracic somites of stage 21–22 embryos ($n = 13$), *Pax-3* (Figure 4k) and *MyoD* (Figure 4l) were lost over several somites, but at the periphery *Pax-3* was upregulated (after 25 hours of re-incubation). Implantation of heparin beads soaked in PBS did not affect the expression of either *Pax-3* or *MyoD* or reveal any acridine orange staining (data not shown).

Figure 4



A graded response to BMP signalling determines positioning of pre-muscle masses in the limb. Beads soaked in high doses of (a,b) BMP-2 (100 μg/ml), (c,d) BMP-4 (10 μg/ml), (e,f) BMP-7 (1 mg/ml) and (g,h) TGF-β1 (10 μg/ml) were inserted into posterior mesenchyme of the right wing and thoracic somites at stages 21–23. The time after manipulation is indicated. Changes in (a,c,e,g) *Pax-3* expression and (b,d,h) *MyoD* expression are indicated by arrows. (f) Before *in situ* hybridisation, the same wing from (e) was incubated in acridine orange which labelled apoptotic cells near the BMP-7 bead (apoptotic cells appear as fluorescent dots; the bead is marked by red dotted circle). Beads soaked in high concentrations of (i,j) BMP-2 and (k,l) BMP-7 were inserted into thoracic somites. Changes in (i,k) *Pax-3* expression and (j,l) *MyoD* expression are indicated by arrows.

Thus, BMP at concentrations that were able to induce apoptosis caused a simultaneous loss of *Pax-3* and *MyoD* expression; this indicates a loss of undifferentiated and

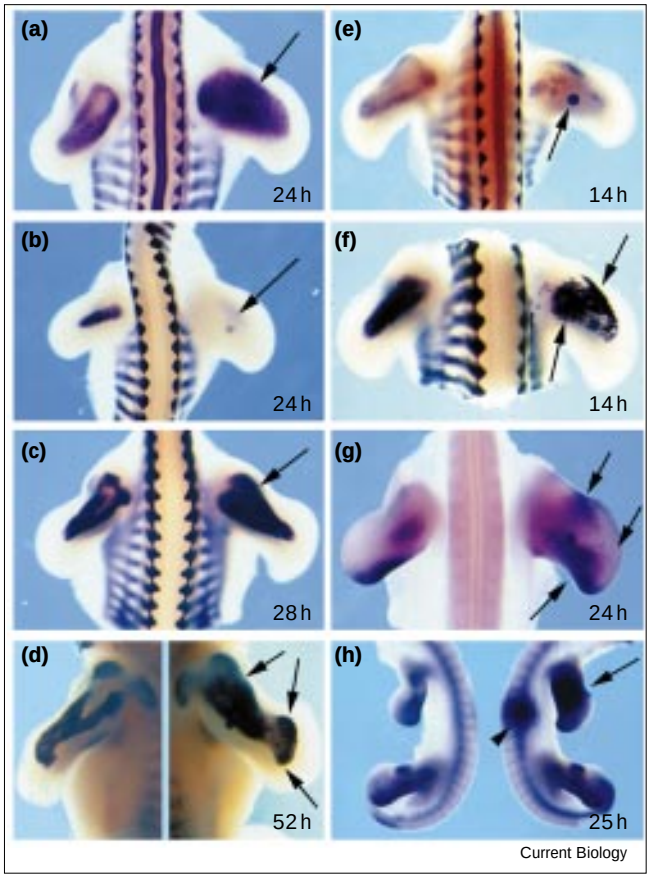
differentiated muscle cells. *Pax-3* was upregulated at some distance from BMP beads but not at this distance away from TGF-β1 beads, however. These results suggest that high BMP concentrations lead to muscle loss by induction of apoptosis and low concentrations increase myogenic proliferation. Thus, a dose-dependent response to BMP spatially co-ordinates the positioning and growth of the pre-muscle masses within the anterior–posterior and proximal–distal limb axes.

Shh stimulates muscle growth in limbs via induction of proliferative signals

Shh in zebrafish has been shown to amplify the number of myogenic cells [24]. It is possible that such a response to Shh could be achieved by the induction of a proliferative signal. Indeed, it is known that Shh can induce *Bmp* expression during limb development [25,26]. We therefore used the limb to examine the activity of Shh on muscle in the presence of ectopically induced proliferative signals. To show that Shh is able to induce *Bmp* in the limb, we inserted beads soaked in Shh protein into the centre of dorsal wing mesenchyme of embryos at stages 21–22 and hybridised with probes against *Bmp-2* and *Bmp-7* transcripts ($n = 5$). Shh bead insertion for 24 hours resulted in an upregulation of *Bmp-2* expression and the *Bmp-2* expression domain was enlarged on the dorsal wing surface (Figure 5g). We examined such a wing in transverse section and detected an upregulation of *Bmp-2* in the dorsal ectoderm and subectodermal mesenchyme (data not shown). Furthermore, in the presence of additional Shh, the distal *Bmp-7* expression domain extended anteriorly and a faint increase of expression at the dorsal limb surface was detected (data not shown).

We then examined the effect of Shh on limb-muscle development. We inserted beads soaked in Shh protein into the centre of dorsal wing mesenchyme of embryos at stages 20–24 and determined the effect on pre-muscle masses by monitoring expression of *Pax-3* ($n = 16$) and *MyoD* ($n = 15$). After 24 hours, *Pax-3* expression was dramatically upregulated in a wing from a stage 20 embryo, and the size of the expression domain was enlarged (Figure 5a). Transverse sections revealed that *Pax-3* expression was confined to a sub-ectodermal layer (data not shown). After an identical procedure, *MyoD* expression was completely inhibited, however (Figure 5b). Insertion of an Shh bead at later stages only partially prevented *MyoD* expression (data not shown). These experiments indicate that myogenic cells that were already expressing *MyoD* at the time of bead insertion did not stop expressing it, but the transition of cells from expressing *Pax-3* to expressing *MyoD* was prevented. Wing buds after exposure to Shh appeared twice the size of non-operated limbs (Figure 5a,b). The area over which Shh stimulated *Pax-3* expression correlated with the area over which *Patched* expression was upregulated ($n = 3$; Figure 5h). Intriguingly, as early as 28 hours after Shh had been applied to stage 24 wings, cells of the enlarged pre-muscle masses differentiated and *MyoD* expression was upregulated (Figure 5c), although high levels of *Pax-3* expression were still maintained. Transverse sections of limbs incubated with Shh and hybridised with probes for *Pax-3* and *MyoD* showed that normal expression sites were enlarged in proportion with the enlargement of the limb. Expression of *Pax-3* and *MyoD* was confined to the dorsal and ventral pre-muscle masses and was never detected at ectopic sites (data not shown). An enlarged *MyoD*-expression domain was still detected 52 hours after Shh beads were applied to a stage 21 wing (Figure 5d). Therefore, in the limb, ectopic Shh

Figure 5



Effect of ectopic Shh on limb muscle development. The time after manipulation is indicated. Arrows show expression domains. An Shh bead was implanted into the wing mesenchyme of an embryo at stage 20 and (a) *Pax-3* expression or (b) *MyoD* expression was assessed. (c) *MyoD* expression 28 h after Shh bead insertion at stage 24. (d) *MyoD* expression 52 h after Shh bead insertion at stage 21. An Shh bead was inserted into the wing mesenchyme after dorsal ectoderm removal at stage 22 and (e) *Pax-3* expression or (f) *MyoD* expression was assessed. (g) An Shh bead was implanted into wing mesenchyme at stage 22 and *Bmp-2* expression assessed. (h) An Shh bead was implanted into the wing mesenchyme and thoracic somites at stage 22 and expression of *Patched* seen in both the limb (arrow) and somites (arrowhead) after 25 h.

upregulated proliferative signals, revealed by expression of *Bmp-2* and *Bmp-7*. Furthermore, Shh upregulated *Pax-3*, delayed *MyoD* expression and, finally, accelerated muscle growth. Thus, Shh and ectoderm have similar effects on limb muscle; this suggests that Shh acts indirectly as a proliferative signal, via pathways induced by the ectoderm and mesenchyme. We therefore tested the effect of Shh on limb muscle after ectoderm removal.

We applied Shh beads to dorsal limb mesenchyme of embryos at stages 22–23 after ectoderm removal and re-incubated the operated embryos for 20 hours ($n = 12$). After this procedure, *Pax-3* expression was downregulated (Figure 5e) and *MyoD* was upregulated dorsally (Figure 5f), whereas ventrally, where the ectoderm was intact, *Pax-3* was upregulated and *MyoD* was downregulated (data not shown). *Patched* expression was broadly upregulated throughout the mesenchyme which clearly indicated the efficacy of Shh in limbs lacking ectoderm (data not shown). Beads soaked in PBS did not disrupt the normal expression of *Pax-3*, *MyoD*, *Bmp-2* or *Patched*. Thus the effect of Shh in inducing *Pax-3* was indirect and mediated via the BMPs.

Discussion

Examining the fate of an embryonic cell lineage *in vivo* and its response to environmental cues is extremely complex, especially when the founder cells are multi-potent and when markers are not available for analysing the undifferentiated and differentiated states of cells. In this study we focused on limb-muscle development, which is an attractive system because the precursor cells are heterotopic in origin and have a single differentiated fate [4]. These precursors apparently have limited developmental options: they can either remain as undifferentiated muscle precursors, differentiate or undergo apoptosis. Furthermore, specific markers exist for myogenic precursors (*Pax-3* [22]) and differentiating muscle cells (*MyoD* [7]). Using the chick limb as an experimental system, we could examine how extrinsic signals influence the fate of limb-muscle cells.

Inhibition of Pax-3 activates MyoD expression and stops further muscle development

Ectoderm signals maintain *Pax-3* expression in cells that form a proliferating muscle-precursor pool. Muscle growth is dependent upon maintaining a balance between undifferentiated proliferative cells and differentiated cells. Removal of ectoderm inhibits muscle growth by depriving precursor cells of the proliferative signals and results in premature differentiation. This exhausts the muscle-precursor pool, leading to eventual muscle loss. Therefore, to understand how embryonic limb muscle mass is generated, the question to address is not only how differentiation is induced but also how muscle progenitors are maintained in a proliferative state.

The correlation of *Pax-3* expression with proliferation and of *MyoD* expression with a post-mitotic state supports the view that expression of *Pax-3* and expression of *MyoD* are mutually exclusive, and suggests that muscle-mass generation is a subdivided process of proliferation and differentiation. During embryonic development, this subdivision is spatially organised, with *Pax-3*-expressing cells situated near the ectoderm and *MyoD*-expressing cells situated deeper in the mesenchyme. Our finding that *Pax-3* expression is linked to proliferation is supported by the observation of *Pax* expression in human tumours. Furthermore, our results confirm the view that members of the *Pax* family play an essential role in determining the size and shape of whole organs [27]. Our experiments do not completely rule out, however, the possibility that there are cells that can simultaneously proliferate and express *MyoD* in the presence of ectoderm. Such cells can only be ones that are in transition between the *Pax-3* layer and the *MyoD* layer, however, and therefore represent a very small portion of the total number of *MyoD*-positive cells. We demonstrated that premature differentiation of limb muscle prevented distal elongation of the muscle masses during further limb outgrowth. Thus, a source of undifferentiated myogenic cells must be maintained so that myogenic cells can migrate, because differentiating muscle cells lose their migratory ability.

A graded response to BMPs spatially co-ordinates muscle growth and position

A striking feature of the early limb bud is the spatial arrangement of *Pax-3* and *MyoD* expression compared with *Bmp* expression (Figure 1t). In all three limb axes, *Bmp* expression domains do not overlap with *Pax-3* expression, but *Pax-3*-expressing cells accumulate preferentially at the border of *Bmp*-expressing tissue. *MyoD*-expressing cells, however, are not found in proximity to *Bmp* domains and are situated deeper in the mesenchyme. We demonstrated that BMP-2, BMP-4 and BMP-7 could be responsible for this organisation. Low concentrations of BMPs maintained *Pax-3* expression after ectoderm removal and resulted in muscle growth. None of the BMPs was as effective as the ectoderm, however. This could be due to our use of homodimeric BMPs, which have been shown to have far lower signalling capacity than naturally occurring heterodimers [28,29]. Mesenchymal BMPs and sub-ectodermal BMPs were unable to maintain *Pax-3* expression, suggesting that these BMPs are in a different biochemical state from ectodermal BMPs and BMPs introduced on beads. It is possible that the ectoderm contains some BMP maturation factor(s). In addition, other ectodermal proliferative signals, such as Wnt proteins, could be acting on muscle [16,30,31]. It is not possible for us to determine the physiological concentrations of BMPs using our approach because homodimers are unlikely to be the natural active agents.

In the presence of beads soaked in high concentration of BMPs, myogenic cells were lost through apoptosis near to

the source, but, remarkably, *Pax-3*-expressing cells accumulated at some distance from the bead. This shows that BMPs act as diffusible morphogens. A threshold response of myogenic cells to different BMP concentrations could divide the limb mesenchyme into three different zones: one zone of high BMP activity, which myogenic cells avoid or in which they die; a second zone of moderate BMP activity, in which myogenic cells preferentially accumulate and proliferate; and a third zone of low BMP activity, which is not sufficient to maintain proliferation and in which myogenic cells can differentiate. Thus, a graded response to BMPs spatially co-ordinates growth and positioning of muscle in all three axes of the limb. Such a concentration-dependent activity of BMPs may explain why, in different situations, BMPs could either induce *Pax-3* expression or induce a loss of all myogenic cells [19,20].

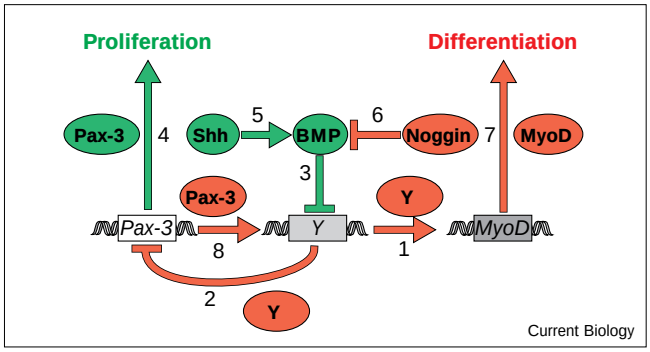
The action of Noggin, a BMP antagonist that is expressed in the mesenchymal core of the limb and thus adjacent to *MyoD* expressing cells, could elaborate this BMP threshold system by directly binding to, and inactivating, BMPs [23]. Thus, a single family of signalling molecules, BMPs, can regulate the crucial events during embryonic muscle growth: when and where to proliferate, and when and where to differentiate. We have also shown that BMP upregulation following Shh application to the limb transiently delays muscle differentiation and stimulates *Pax-3* expression, eventually leading to unco-ordinated and excessive muscle growth. We were able to rule out a direct link between Shh and *Pax-3* expression by applying Shh to limbs after ectoderm removal; Shh did not have any effect on *Pax-3* expression.

Is muscle differentiation a 'default' pathway?

Our results strongly indicate that any pathway which downregulates *Pax-3* expression forces myogenic cells to express *MyoD* and thus to differentiate. Overexpression of *Pax-3* can, however, transform some but not all non-muscle cell lines into a muscle lineage, suggesting that *Pax-3* can act as an activator of *MyoD* [30]. To resolve this apparent contradiction, we have to take into account other observations. Head muscle activates *MyoD* expression without having expressed *Pax-3* [32]. Likewise, dispersed epiblast cells will express *MyoD* after a 4 hour culture period although they have never expressed *Pax-3* or the muscle-determination factor Myf-5 [8]. Furthermore, some *MyoD*-expressing cells have been detected in the trunk of mice that have a mutated *Pax-3* gene (Splotch mutants) and also lack Myf-5, although in these mutants almost all trunk, limb and tongue muscles are missing [32]. Thus, muscle differentiation can occur independently of *Pax-3* activation.

We propose a model (Figure 6) that integrates all the above findings and accommodates our suggestion that

Figure 6



How different signalling molecules could influence myogenic proliferation (in green) and differentiation (in red) through regulation of a competence factor (Y). See text for further details.

muscle differentiation is initiated through a default pathway. We suggest that a competence factor (Y), which is a transcription factor, resides upstream of *MyoD*. When factor Y is active, it can initiate *MyoD* expression (arrow labelled 1 in Figure 6), but it also represses *Pax-3* expression (2) and therefore stops proliferation and induces muscle differentiation. BMPs and other proliferative (or *Pax-3*-inducing) signals, such as Wnt [30,31], repress *Y* expression (3) and so prevent premature muscle differentiation. Repression of *Y* expression removes repression of *Pax-3* and activates proliferation (4). Shh induces BMP (5), thereby activating proliferation. The repression of *Y* by BMPs can be lifted by Noggin (6), leading to an upregulation of *MyoD* expression and initiation of differentiation (7) and a repression of *Pax-3* and proliferation. In our model, however, the removal of repressing signals is sufficient to upregulate *Y* and *MyoD* expression, and inducing signals are not necessary to initiate differentiation in determined myogenic cells. *Pax-3* itself is upstream of *Y* and can activate *Y* expression (8) which explains how proliferative *Pax-3*-expressing myogenic cells can maintain their determined state. The potential of *Pax-3* to induce *Y* expression and, subsequently, *MyoD* expression is seen when cells are transfected with *Pax-3*, which results in *MyoD* activation [30].

Conclusions

We have shown that continuous limb-muscle growth requires the maintenance of myogenic proliferation and the repression of muscle differentiation. Proliferation is strongly linked to *Pax-3* expression but is excluded by *MyoD* expression and differentiation. BMPs act in a dose-dependent manner, either as proliferative signals or to prevent muscle development, restricting limb-muscle growth to defined positions. Muscle differentiation can occur merely after cells have escaped the influence of proliferative signals, as shown by the effect of ectoderm removal. Additionally BMP may upregulate *Noggin*, which

encodes a BMP antagonist (H.A., B.C., K.P., unpublished observations). Noggin might restrict the proliferative action of BMPs, and thus promote differentiation. Our results indicate that muscle differentiation is controlled by regulating and modulating proliferative signals but can be autonomously initiated through a default pathway. BMPs and functionally related molecules might be able to co-ordinate muscle growth in space and time. An imbalance of BMPs and related factors alters the onset of differentiation, and muscle growth is either arrested when differentiation is prematurely initiated or is excessive when differentiation is delayed.

Materials and methods

Preparation of chick embryos

Fertilised chicken eggs were incubated at 38°C, and the embryos were staged according to Hamburger and Hamilton [33]. Experiments were performed on embryos at stage 20–24. The embryos were re-incubated for 6 days, sacrificed, and processed for whole-mount *in situ* hybridisation or antibody staining.

Bead implanting and microsurgical procedures

BMP-2 and BMP-4 were provided by Genetics Institute. Shh was a gift from Andy McMahon. BMP-7 was obtained from ICRF. TGF-β1 was from R&D Systems. All proteins were applied to 80–120 μm beads. Shh was applied to Affigel beads (Sigma) and BMP-2, BMP-4 and BMP-7 were applied to heparin acrylic beads (Sigma). Shh was used at 14 mg/ml. BMPs were used at concentrations stated in text. Proteins were loaded onto beads as described [34]. For bead implant, the dorsal ectoderm and mesenchyme of the right wing or somites were punctured with an electrolytically sharpened tungsten needle, and a bead was inserted into the punctured mesenchyme using a blunt glass needle. For ectoderm removal, the ectoderm was stained with nile blue *in ovo* using a blunt glass needle coated with 2.5% agar containing 2% nile blue. The ectoderm was peeled from the mesenchyme immediately after staining. At limb level, ectoderm was removed only from the dorsal side, avoiding the limb margins, apical ectodermal ridge and the ectoderm overlying the zone of polarising activity. For ectoderm transplantation, stage 28 donor embryos were sacrificed, transferred to DMEM medium (Sigma), the dorsal half of the wing stylopod dissected and incubated in 2% trypsin/PBS (Sigma) for 1 h at 4°C. Trypsinised limb pieces were transferred back into medium and the ectoderm peeled from the mesenchyme. A small slit was made into the dorsal ectoderm and mesenchyme of the right wing of the host embryo (stage 20) in which a small piece of ectoderm was manipulated.

Histology assays

Acridine orange was used to determine apoptosis as the reagent has been shown to be specifically intercalated by cells undergoing programmed cell death and not by those dying via necrosis [35]. Wing buds were dissected free of all unwanted tissue and incubated in acridine orange (100 ng/ml in PBS) at 37°C for 30 min. Specimens were washed 2×2 min in PBS and flattened on a microscope slide under a coverslip [36]. Samples were photographed immediately using fluorescence illumination and fixed for *in situ* hybridisation.

For whole-mount *in situ* hybridisation, all chick embryos were washed in PBS and fixed overnight in 4% paraformaldehyde at 4°C. Antisense RNA probes were labelled with either digoxigenin or fluorescein, and whole-mount *in situ* hybridisation was performed as described [37]. The following probes were used in this study: *Bmp-2*, PCR-cloned fragment (nucleotides 1–797); *Bmp-4*, PCR-cloned fragment (nucleotides 1–953; gift from Paul Brickell); full-length *Bmp-7* (1.1 kb; gift from Antony Graham); full-length *MyoD*, clone CMD9 (1.5 kb; gift from Bruce Patterson); full length *Noggin* (~700 bp; gift from Jonathan Cooke); *Patched*, PCR-cloned fragment (900 bp; gift from Cliff Tabin); *Pax-3*, 645 bp fragment corresponding to nucleotides 468–1113 (gift from Martin Goulding). Whole-mount embryos were cryo-sectioned for further histological examination.

For BrdU labelling, 30 min before fixation, 100 μl 40 mM BrdU (Sigma) dissolved in water was added on the vitelline membrane and embryos were re-incubated at 38°C. Embryos were fixed overnight in Serra solution (60% ethanol, 30% formaldehyde, 10% glacial acetic acid) at 4°C, dehydrated, wax-embedded and sectioned. Antibody staining was preceded by hydrolysis for 30 min with 2 N HCl. Immunohistochemistry was performed by indirect immunoperoxidase method with monoclonal anti-BrdU antibody (DAKO) and peroxidase-conjugated goat anti-mouse immunoglobulin (IgG; Sigma) as second antibody. Diaminobenzidine (DAB) was used as chromogen, and weak counter-staining was performed with true red.

For immunohistochemistry, embryos were fixed in Serra solution, wax-embedded and serially sectioned at 8 μm. Sections were labelled with either a desmin monoclonal antibody (1:100; DAKO) or with a muscle-specific actin monoclonal antibody (1:5000; Sigma). The secondary antibody was a peroxidase-conjugated goat anti-mouse IgG antibody (1:300; Sigma). DAB was used as a chromogen. For resin histology, embryos were fixed in 0.12 M sodium cacodylate solution containing 3% glutaldehyde and 2% formaldehyde. They were embedded in epon resin (Serva) according standard procedure and sections at 0.75 μm using a Leitz-Ultra-Cut-S microtome. For Feulgen staining, wax-embedded sections (8 μm) were stained according to standard procedure (light green and Schiff's Reagent).

Control experiments

We inserted beads soaked in either PBS only, BMP-2 at 100 ng/ml or inactivated Shh. Ectoderm was either stained with nile blue but not removed or peeled off, but positioned back on the mesenchyme. These embryos were hybridised with probes against *Pax-3* or *MyoD*; none of the controls influenced the expression of either gene.

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